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# Context-dependent tree species effects on soil nitrogen transformations and related microbial functional genes

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**Abstract** Although it is generally accepted that tree species can influence nutrient cycling processes in soils, effects are not consistently found, nor are the mechanisms behind tree species effects well understood. Our objectives were to gain insights into the mechanism(s) underlying the effects of tree species on soil nitrogen cycling processes, and to determine the consistency of tree species effects across sites. We compared N cycling in soils beneath six tree species (ash, sycamore maple, lime, beech, pedunculate oak,

Norway spruce) in common garden experiments planted 42 years earlier at three sites in Denmark with distinct land-use histories (forest and agriculture). We measured: (1) net and gross rates of N transformations using the  $^{15}\text{N}$  isotope pool-dilution method, (2) soil microbial community composition through qPCR of fungal *ITS*, bacterial and archaeal *16S*, and (3) abundance of functional genes associated with N cycling processes—for nitrification the archaeal and bacterial ammonia-monooxygenase genes (*amoA* AOA and *amoA* AOB, respectively) and for denitrification, the nitrate reductase genes *nirK* and *nirS*. Carbon concentrations were higher in soils under spruce than under broadleaves, so N transformation rates were standardized per g soil C. Soil  $\text{NH}_4^+$  parameters (gross ammonification, gross  $\text{NH}_4^+$

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consumption, net ammonification (net immobilization in this case), and  $\text{NH}_4^+$  concentrations, per g C) were all lowest in soils under spruce. Soils under spruce also had the lowest gene abundance of bacteria, bacterial:fungal ratio, denitrifying microorganisms, ammonia-oxidizing archaea and ammonia-oxidizing bacteria. Differences in N-cycling processes and organisms among the five broadleaf species were smaller. The ‘spruce effect’ on soil microbes and N transformations appeared to be driven by its acidifying effect on soil and tighter N cycling, which occurred at the previously forested sites but not at the previously agricultural site. We conclude that existing characteristics of soils, including those resulting from previous land use, mediate the effects of tree species on the soil microbial communities and activities that determine rates of N-cycling processes.

**Keywords**  $^{15}\text{N}$  isotope pool-dilution · N transformation rates · Soil microbial communities · qPCR · Tree species

## Introduction

Although differences in soil properties have often been reported under different tree species, our ability to generalize about the expected influence of a given tree species has been impaired by the inconsistency of tree species effects. Although the effects of a given species are generally consistent when detected, studies that examine tree species effects across multiple sites have often reported differences in the magnitude of effects among sites (Alban 1969; Klemmedson 1995; Binkley and Giardina 1998; van Oijen et al. 2005; Neirynck et al. 2000; De Schrijver et al. 2007). With respect to N cycling, Priha and Smolander (1999) found that influences of pine, spruce and birch on soil N transformations differed at two sites of contrasting fertility. Differences in rates of net mineralization and nitrification rates among four coniferous tree species were detected at two fertile valley-bottom sites, but not at less fertile, mid-slope sites (Prescott et al. 2000). Examining data from 35 studies in temperate and boreal forests, Mueller et al. (2012a, b) found that tree species and plant traits had strong impacts on net N mineralization, but effects were not consistent across sites, which they attributed to the modulating effects

of other site factors such as stand age and soil type. Recognition of the context-dependent nature of the tree-species influence on soils prompted Prescott and Vesterdal (2013) to suggest that research questions be refined to clarify the conditions under which the effect of a given tree species will be evident.

The mechanisms through which tree species influence N cycling processes in soil appear to be manifold. Rates of net N mineralization and nitrification have been correlated with N concentrations (or C:N ratio) of leaf litter (Gower and Son 1992; See et al. 2015), forest floor (Priha and Smolander 1999; Prescott et al. 2000), and soil (Finzi et al. 1998; Lovett et al. 2004; Ross et al. 2004). Other studies of tree species effects have reported stronger influences of factors related to acidity and base status (Grayston and Prescott 2005; De Schrijver et al. 2007; Hobbie et al. 2007), which have been attributed to differences among tree species in concentrations of base cations in leaf litter (Reich et al. 2005). These differences may also be related to the mycorrhizal association of tree species, as tree species with arbuscular mycorrhizal associations tend to maintain high base saturation and pH while those with ectomycorrhizal association tend to acidify soil (Phillips et al. 2013). Effects of acidity and base status may also be mediated through effects on the abundance of burrowing earthworms (Reich et al. 2005), which create profound differences in the properties of the topsoil (Schelfhout et al. 2017) and the microbial community, particularly bacterial: fungi ratio (Rousk et al. 2009). Tree species-mediated differences in soil pH have a particularly strong effect on rates of nitrification processes (Priha and Smolander 1999; Rousk et al. 2010), attributable to the sensitivity of ammonia-oxidizing bacteria (AOB) to soil pH (Nicol et al. 2008).

In Denmark, a common garden experiment with six common European tree species replicated across several sites has been used to explore tree species effects on soil properties and C and N cycling processes. Three decades after planting, the six species formed three distinct groups according to effects on litterfall, forest floor and soil attributes (Vesterdal et al. 2008, 2012; Schelfhout et al. 2017): (1) ash (*Fraxinus excelsior* L.), sycamore maple (*Acer pseudoplatanus* L.) and small-leaved lime (*Tilia cordata* L.), (2) beech (*Fagus sylvatica* L.) and pedunculate oak (*Quercus robur* L.), and (3) Norway spruce (*Picea abies* (L.) Karst.). Ash, maple and lime plots were

characterized by leaf litter rich in N, P and base cations, faster rates of leaf-litter decomposition and forest-floor turnover, high soil pH, C and N, and greatest abundance and diversity of burrowing earthworms. In comparison, spruce plots had high litter C/N, large accumulations of C and N in forest floor, slow litter decomposition, low soil pH, low base cation concentrations, and low abundance of earthworms, especially burrowing types. Beech and oak plots were intermediate to these extremes. Despite the consistency of these tree species effects, variation in the expression of tree species effects on soils among the study sites has been noted. Gurmesa et al. (2013) found differences in soil nitrate concentrations among tree species growing on fine-textured and fertile soils but concentrations were negligible under all species on sandy acidic soils. Christiansen et al. (2010) attributed the lack of significant differences among tree species in rates of potential net N mineralization, nitrification and N leaching to a consistently large site effect when comparing two of the sites—one that was formerly a beech forest and the other which had been under agriculture for more than a century.

In this study we revisit three of these Danish common garden experiments to assess the influence of these six common European tree species on soil N transformations. We measure gross rates of N transformations (production and consumption of ammonium and nitrate) and abundance of functional genetic markers, i.e. genes encoding for enzymes specifically involved in N-transformation processes such as ammonification, nitrification and denitrification (Levy-Booth et al. 2014). This combination of functional genes and gross N transformation pathways surpass what can be gleaned from traditional N mineralization incubations and microbial biomass estimation techniques, by specifically linking processes with functional soil microbial communities (Levy-Booth et al. 2014; Ribbons et al. 2016). We focused on exploring the mechanisms that underlie these tree species effects at the soil layer scale.

We addressed the following specific questions: (1) Do soils associated with common European tree species differ in soil N transformation rates? (2) Do tree species influence abundances of functional genes related to N transformations? (3) Which mechanisms underlie the influences of these tree species on soils? (4) Are tree species effects consistent or variable among sites? Consistent with the functional species

groups identified in earlier studies of these experiments (Vesterdal et al. 2008, 2012; Christiansen et al. 2010; Gurmesa et al. 2013), we hypothesized that the six common European tree species would also form three distinct clusters in terms of N transformations and functional genes: soils under ash, maple and lime would have higher N transformation rates (especially nitrification), abundance of associated functional genes and bacterial:fungal ratios than soils under oak and beech, which would again be higher in these traits than soils under Norway spruce.

## Methods

### Study site and sampling scheme

Three sites were selected from the Danish tree species trials that were established in 1973 as monoculture 0.25-ha stands of each tree species, across a pH and soil nutrient gradient (Vesterdal et al. 2008). Mattrup had an agricultural land-use history, whereas both Vallø and Viemose were previously forested sites (beech forest).

Site Mattrup is located in Ring Skov on the peninsula of Jutland in the western part of Denmark (55°N57'36", 9°E37'10"). The elevation is 100–110 m asl. and the site gently slopes eastward. The soil is a loamy till and is characterized as a well-drained, mixed, acid, mesic, coarse-loamy Lamellic Hapludalf (Soil Survey Staff 2014). The mean annual temperature in the period 2005–2015 was 8.5 °C with mean annual precipitation of 829 mm (Danish Meteorological Institute 2005). Mattrup was agricultural land from 1842 to 1973, thus a period of at least 130 years and probably longer (Christiansen et al. 2010).

Site Vallø is located on the island of Zealand (55°N25'23", 12°E3'47") 40 km southwest of Copenhagen at an elevation of 50 m asl. and gently sloping westward. The soil is a glacial till and is characterized as a moderately well-drained, mixed, nonacid, mesic, coarse-loamy Oxyaquic Hapludalf (Soil Survey Staff 2014). The mean annual temperature in the period 2005–2015 was 8.8 °C with a mean annual precipitation of 635 mm (Danish Meteorological Institute 2005). Vallø has been a managed beech forest for the last 250 years (Christiansen et al. 2010).

The third site, Viemose, is located at the coast in the southeast corner of the island Zealand (55°N1'2",

12°E9'27") at an elevation of 10 m asl. The soil is a glacial till and is characterized as a well-drained, fine-loamy, mixed, acid Typic Hapludalf (Soil Survey Staff 2014). The mean annual temperature in the period 2005–2015 was 9.2 °C with a mean annual precipitation of 618 mm (Danish Meteorological Institute 2005). Viemose has been a managed forest for at least 250 years (Danish Cadastre 2017), and the tree species was beech for more than a century before the experiment was established.

Monoculture stands (0.25 ha, within a total area of 1.6 ha) of common ash, European beech, pedunculate oak, small-leaved lime, sycamore maple and Norway spruce were planted in 1973 as part of a national tree species experiment (Vesterdal et al. 2008). There was no replication of tree species within each site so the sites serve to ensure replication. The original beech trees at Vallø and Viemose were felled at the establishment of the plots. The understory was not managed (i.e. removed) at the sites and was most abundant in the more open ash and oak stands where ground vegetation (herbs, grasses and some seedlings and saplings) covered the forest floor. Ground vegetation was almost non-existent in spruce, beech and lime stands apart from transient spring flora. Individual tree species plots had been thinned approximately every fourth year since 1987 and had been managed according to common practice (spacing and thinning) of the forest districts (Christiansen et al. 2010). Ash was absent at Vallø due to establishment failure.

In June 2015, four mineral soil cores were taken from 0 to 10 cm depth in the cardinal points of three 5-m radius subplots within each plot (see Fig. S1 for sampling scheme). Samples from within each subplot were composited in the field yielding three replicate soil samples for each tree species per site ( $n = 3$ ). Soil samples were placed in coolers in the field and transported back to the University of Copenhagen for subsequent analyses where they were stored at 4 °C for 8 h. Soils were sieved through a 2-mm mesh sieve and six 15-g subsamples were immediately removed for the  $^{15}\text{N}$  pool-dilution analyses. A portion of each composite sample was stored at  $-20$  °C for DNA extraction, and tools were sterilized with ethanol between samples to minimize cross-contamination.

## Soil C, N and pH

Total organic C and N concentrations were determined on ground samples by dry combustion based on the Dumas method (Matejovic 1993) using a FLASH 2000 EA NC Analyzer (Thermo Fisher Scientific, Waltham, MA, USA) with a quantification limit of 0.07% C. Soil pH (< 2 mm fraction) was determined in solution of 0.01 M  $\text{CaCl}_2$  (1:2.5 soil-solution ratio) and analysed with a combination electrode GK2401 (Radiometer, Copenhagen, Denmark). No major differences in soil bulk density, water holding capacity, and stone content were observed in previous studies within these same plots (Vesterdal et al. 2008).

## Gross rates of N ammonification and nitrification

The  $^{15}\text{N}$  pool-dilution method, modified through the use of plastic Nalgene jars in place of glass mason jars used by Drury et al. (2008) and Hart et al. (1994), was used to determine gross rates of N ammonification and nitrification, with each soil sample analysed in triplicate. This method adds a fixed quantity of  $^{15}\text{N}$  for a specific soil, and does not constitute a major fertilization effect at our sites in which the inputs of N with throughfall are higher than in the location where this method was developed in North America. Six 15 g subsamples from each soil sample were transferred to 250 ml Nalgene jars and sealed with Parafilm ( $n = 3$  subsamples for both  $^{15}\text{NH}_4^+$  and  $^{15}\text{NO}_3^-$ ). The Parafilm seal was punctured to enable gas exchange and maintain aerobic conditions throughout the experiment, and samples were then incubated at room temperature for 24 h prior to initial  $^{15}\text{N}$  fertilization treatments. Four ml of  $^{15}\text{NH}_4\text{Cl}$  solution (99 atom %; Sigma Aldrich) or 4 ml of  $\text{K}^{15}\text{NO}_3$  (99 atom %; Sigma Aldrich) was added to the soil samples in each respective jar, with an equivalent application rate of  $12 \mu\text{g N g}^{-1}$  soil. This rate would be equivalent to addition of around  $1 \text{ kg N ha}^{-1}$  to the top 1 cm of mineral soil. For comparison, the annual mean N throughfall flux ( $\text{kg N ha}^{-1} \text{ year}^{-1}$ , SE,  $n = 4$ ) at Vallø and Mattrup sites for spruce ( $28 \pm 2$ ) was significantly larger than for maple ( $12 \pm 1$ ), beech ( $11 \pm 1$ ) and oak ( $9 \pm 1$ ) stands but not different from that of lime ( $15 \pm 3$ ) (Christiansen et al. 2010). Labelled N was injected into the samples in 1 ml intervals four times, and gently homogenized by hand-mixing to ensure isotopic labelled N was applied

uniformly throughout the soil sample, and the Parafilm seal was replaced.

A 5 g soil subsample was removed from each Nalgene jar and placed into a 50 ml falcon tube, immediately after  $^{15}\text{N}$  fertilization, 24 h after  $^{15}\text{N}$  fertilization, and 48 h after  $^{15}\text{N}$  fertilization. Fifty ml of 1.0 M KCl was added to each falcon tube, shaken for 1 h, and filtered through glass fibre filters in a vacuum syringe filtration system. A 15 ml subsample of each extract was reserved for ammonium and nitrate concentration analyses, which were determined colorimetrically using a flow injection analyser (FIA PE FIAS 300, Perkin-Elmer, Waltham, MA, USA) using the indophenol-blue and cadmium reduction methods for  $\text{NH}_4\text{-N}$  and  $\text{NO}_3\text{-N}$ , respectively. Due to high anticipated levels of N we prepared  $10 \times$  dilutions of each KCl extract. The remainders of the KCl extracts were used for microdiffusion acid trapping of  $^{15}\text{NH}_4\text{-N}$  and  $^{15}\text{NO}_3\text{-N}$ , with the sequential addition of MgO and Devarda's alloy following the International Atomic Energy Agency protocol (IAEA, 2001). Acid traps were dried, packaged in tin cups, and analysed by elemental flash combustion analysis (EA 1110, Thermo Scientific, Bremen, Germany) in combination with stable isotope ratio mass spectrometry (Delta PLUS, Thermo Scientific, Bremen, Germany).

Net rates of ammonification and nitrification were calculated as the difference in inorganic N between the incubated samples and the initial soil extractions. The gross rates of ammonification, nitrification and microbial consumption were calculated following Hart et al. (1994), such that gross ammonification is equal to:  $((\text{Initial NH}_4^+ \text{ concentration} - \text{Final NH}_4^+ \text{ concentration}) / \text{extraction time}) \times [(\log(\text{APE } ^{15}\text{N Initial} / \text{APE } ^{15}\text{N Final}) / \log(\text{Initial NH}_4^+ \text{ concentration} / \text{Final NH}_4^+ \text{ concentration}))]$ , where APE = Atom Percent Excess. Gross consumption was calculated as:  $\text{Gross ammonification} - ((\text{Final NH}_4^+ \text{ concentration} - \text{Initial NH}_4^+ \text{ concentration}) / \text{extraction time})$ . Net ammonification was calculated as  $\text{Gross ammonification} - \text{Gross consumption}$ . Nitrification rates were similarly calculated. Based on the strong covariance with soil organic matter concentrations, which varied among tree species, N transformation rates were expressed per g C to evaluate the differences in transformation rate dynamics among tree species, and gene copies were similarly expressed per g C.

## DNA extraction, amplification, and quantification

DNA was extracted from 0.25 g of field-moist mineral soil using the MoBio Power Soil DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA). DNA quality and concentration was measured using a nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE) and electrophoresis in 1% agarose gels, then stored at  $-20^\circ\text{C}$  prior to amplification using the primers listed in Table 1. All qPCR runs were completed in duplicate using 20  $\mu\text{l}$  reactions consisting of: 10.0  $\mu\text{l}$  of SYBRGreen (2 $\times$ ) PCR Master Mix (Life Technologies Corp., Carlsbad, CA, USA), 0.25  $\mu\text{l}$  of each forward and reverse primer, 1  $\mu\text{l}$  of DNA template, and 8.5  $\mu\text{l}$  of nuclease-free water. All reactions were run on a Stratagene Mx3005P Real-Time PCR system (Stratagene, La Jolla CA, USA), with fluorescence measured during extension. The specificity of the PCR amplification was tested via the inspection of the melting curves that were prepared at the end of each PCR run. PCR products were also run on a gel to verify the presence of a single band of the correct size.

qPCR conditions for fungal *ITS* were  $95^\circ\text{C}$  for 10 min followed by 40 cycles of:  $95^\circ\text{C}$  for 30 s,  $55^\circ\text{C}$  for 30 s, and  $72^\circ\text{C}$  for 30 s. Standard curves for fungal *ITS* were constructed using ten-fold serial dilutions of purified PCR products containing the *ITS1* region of *Fusarium avenaceum* genomic DNA, which ranged from  $10^3$  to  $10^9$  gene copies. qPCR conditions for bacterial and archaeal *16S* were  $95^\circ\text{C}$  for 10 min followed by 40 cycles of:  $95^\circ\text{C}$  for 15 s,  $53^\circ\text{C}$  for 30 s, and  $72^\circ\text{C}$  for 20 s, and we used ten-fold serial dilutions of purified PCR products containing the *16S* region of *Pseudomonas putida* genomic DNA, which ranged from  $10^2$  to  $10^7$  gene copies.

We quantified Cd-nitrite reductase (*nirS*) and Cu nitrite reductase (*nirK*) with qPCR conditions of 10 min at  $95^\circ\text{C}$  and 40 cycles of:  $95^\circ\text{C}$  for 1 min,  $60^\circ\text{C}$  for 1 min and  $72^\circ\text{C}$  for 1 min. The standard curve for *nirS* and *nirK* used tenfold serial dilutions of  $10^1$ – $10^7$  gene copies from *Pseudomonas putida*. All gene copies were calculated using exact soil extraction weights, and are presented in analyses as log gene copies/ $\mu\text{l}$ /g of dry weight soil.

We quantified the number of *amoA* gene copies for bacterial ammonia oxidizers (*amoA* AOB) and archaeal ammonia oxidizers (*amoA* AOA). These



**Table 1** Group-specific primers for qPCR gene quantification assays

Primer target	Primers	Primer sequence (5'–3')	References
All Bacteria	Eub338	ACT CCT ACG GGA GGC AGC AG	Fierer et al. (2009)
(16S rRNA)	Eub518	ATT ACC GCG GCT GCT GG	
All Fungi (ITS)	BITS	ACCTGCGGARGGATCA	Bokulich and Mills (2013)
	B58S3	GAGATCCRTTGYTRAAAGTT	
AOA <sup>a</sup> ( <i>amoA</i> )	<i>amoA</i> -23F	ATG GTC TGG CTW AGA CG	Francis et al. (2005)
	<i>amoA</i> - 616R	GCC ATC CAT CTG TAT GTC CA	
AOB <sup>b</sup> ( <i>amoA</i> )	<i>amoA</i> -1F	GGG GTT TCT ACT GGT GGT	Rotthauwe et al. (1997)
	<i>amoA</i> -2R	CCC CTC KGS AAA GCC TTC TTC	
Cd-nitrite reductase ( <i>nirS</i> )	<i>nirS</i> -1F	CCT AYT GGC CGG CRC ART	Braker et al. (1998)
	<i>nirS</i> -3R	GCC GCC GTC RTG VAG GAA	
Cu-nitrite reductase ( <i>nirK</i> )	<i>nirK</i> -1F	GGG CAT GAA CGG CGC GCT CAT GGT G	Braker et al. (1998)
	<i>nirK</i> -1R	CGG GTT GGC GAA CTT GCC GGT GGT C	

<sup>a</sup>Ammonia-Oxidizing Archaea<sup>b</sup>Ammonia-Oxidizing Bacteria

primers generate amplification products of 491 bp for bacterial *amoA* and 440 bp for archaeal *amoA*, respectively. The qPCR amplification was performed at 95 °C for 10 min followed by 40 cycles of: 95 °C for 30 s, 57 °C/58 °C for 30 s and 72 °C for 60 s/45 s (AOA/AOB), and concluded with a melting curve analysis. The standard curves for AOA and AOB *amoA* were prepared from the fosmid clone 54d9 and the pCR 2.1-TOPO plasmid carrying AOB *amoA* from *Nitrosomonas europaea* ATCC19718 (Feld et al. 2015), using the primers *amoA*-1F and *amoA*-2R. Standard curves were constructed from the extracted plasmids using tenfold dilutions of 10<sup>1</sup>–10<sup>7</sup> gene copies AOB *amoA* copies per microliter, and 10<sup>1</sup>–10<sup>6</sup> gene copies AOA *amoA* copies per microliter.

### Statistical analyses

We used a two-way analysis of variance (ANOVA), to test for differences in soil properties (pH, C%, N% and C:N) between tree species and sites (using replicate sample averages, such that there were 3 soil replicates and further two 2 technical replicates for all instrumental analyses of response variables within each of the three sites). We did not include an interaction term, as there was no replication of tree species plots within sites (see Supplementary Material for R code). We used Tukey tests and least square means to make paired comparisons between the six species. Overall

effects of tree species on N transformation rates and soil microbial abundances were tested using linear mixed modes. We ran separate models for each response variable and included tree species as fixed factor and site as random factor. We used Tukey HSD for pairwise comparisons between tree species.

We explored relationships between site and soil properties, soil microbial gene abundances and N transformation rates in two ways. First, we used principal component analyses (PCA) to visualize the relationships between the three groups of data: gross and net N transformation rates, all six gene abundances, soil pH and soil C:N ratios. Another PCA was constructed to examine relationships between all six gene abundances, soil pH, and soil C:N ratios. Second, to examine whether tree species effects on microbial abundances were dependent on pH (based on the strong correlation with PC1 in the PCA), we used linear mixed models including tree species, pH and their interaction as fixed effects and site as random. We also explored similar relationships with C:N ratios and N transformation rates as well as gene abundances, which were less significant than pH relationships and are shown in the Supplementary Material section (and all versions of these figures with data presented at per g soil in Supplementary Material). Because of the large number of response and explanatory variables; and because not all relationships between them have a theoretical support, we only

ran these analyses to test certain biologically meaningful relations (e.g. AOA and AOB with gross nitrification; focusing on C:N ratio and pH effects on N transformation rates and gene abundances in subsequent analyses). All analyses were completed in R version 3.3.1 (R Foundation for Statistical Computing 2016) using the *vegan* (Oksanen et al. 2013), *MASS* (Venables and Ripley 2002) and *lmer* (Bates et al. 2014) packages for the mixed effects models and principal components analyses, the *ggplot2* (Wickham 2009) package for all bar charts, *lsmeans* (Lenth 2016) for Tukey's post hoc comparisons. For linear mixed models all solid lines indicate significant relationships, and dashed lines indicate non-significant relationships.

## Results

Soils under ash, lime and maple had the highest pH (> 4) while spruce had the lowest (3.3; Table 2). Soil under spruce had significantly higher concentrations of C and N and wider C:N ratio than soils under the broadleaf species. The wider C:N ratios were similar to those in previous soil samples from these plots (Vesterdal et al. 2008), but higher C and N concentrations were unexpected, and may have resulted from imperfect separation of the organic and mineral layers in the spruce plots. To distinguish between effects resulting from differences in N-cycling and those related only to the amount of organic matter, the data are presented on a per g soil C basis. Among sites, pH

was highest at Matstrup and lowest at Vallø; Vallø also had significantly higher C and N concentrations and C:N ratio than the other sites.

All soil  $\text{NH}_4^+$  variables measured (gross ammonification, gross  $\text{NH}_4^+$  consumption, net ammonification (net immobilization in this case), and  $\text{NH}_4^+$  concentrations) were lowest in the soils under spruce, although differences were not always significant (Fig. 1a–d). Among the broadleaf species, lime had the lowest rates of most of these  $\text{NH}_4^+$  variables. Ash soils had the highest gross rates of ammonium production and consumption and net ammonification (immobilization in this case). There were no significant differences among the tree species in  $\text{NO}_3^-$  concentrations or net nitrification rates (Fig. 1b–d), but spruce was again lowest and ash highest in  $\text{NO}_3^-$  concentrations. Gross nitrification and  $\text{NO}_3^-$  consumption rates were highest in oak and spruce soils, and lowest in lime soils ( $P < 0.01$ ; Fig. 1f, h). Tree species effects were quite similar on a per g soil basis, but more OM (%C) in spruce compensated for lower  $\text{NH}_4^+$  and  $\text{NO}_3^-$  per g C, so spruce was more similar to the other species (Fig. S6).

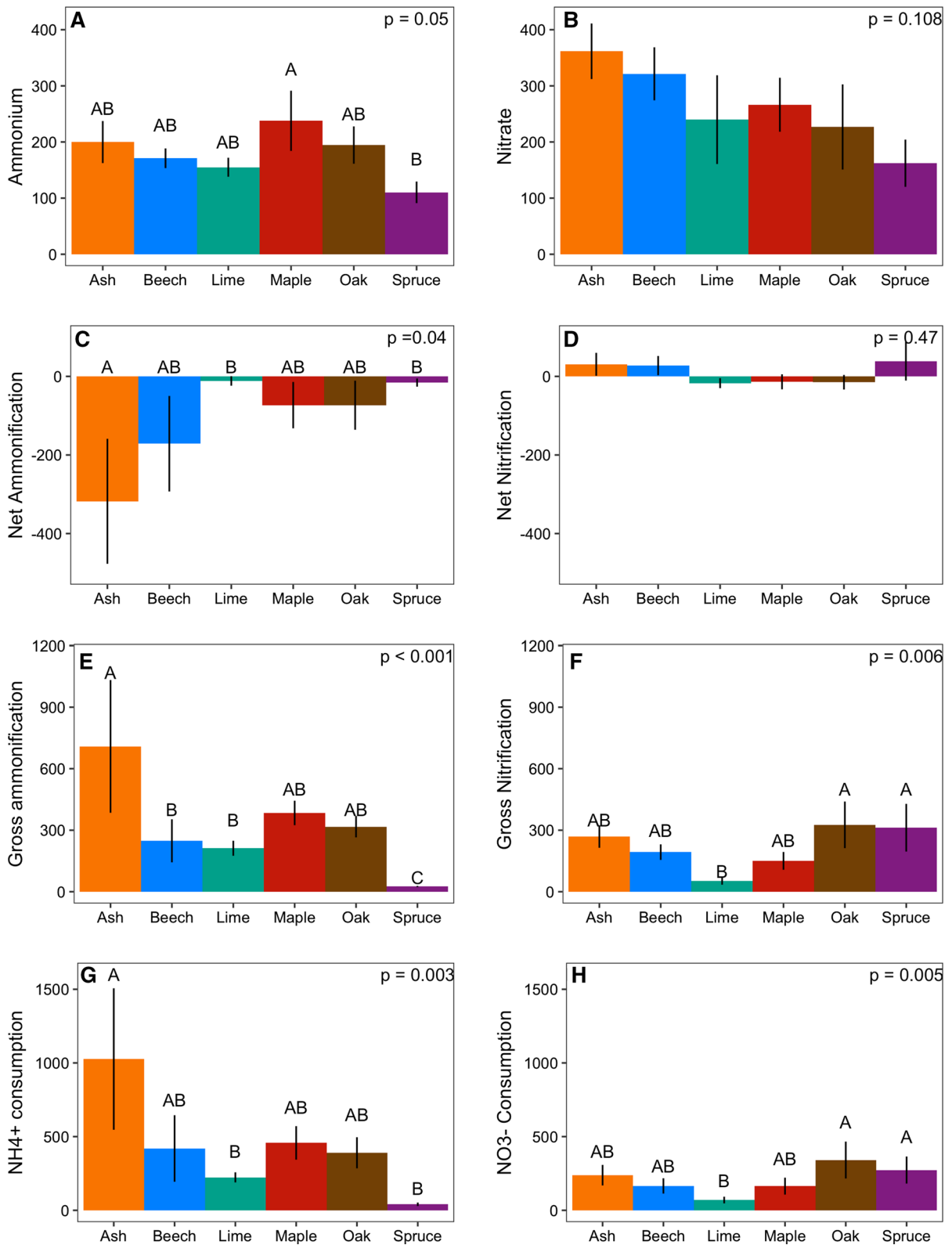
Abundances of microbial functional genes (other than fungal ITS) were lowest and most variable in soils beneath Norway spruce (Fig. 2a–f). Soils under spruce had the lowest gene abundance of bacteria and archaea (16S), denitrifying microorganisms (*nirS*, and *nirK*), ammonia-oxidizing archaea (AOA *amoA*) and ammonia-oxidizing bacteria (AOB *amoA*). Fungal:bacterial ratio (ITS:16S ratio tended to be highest in spruce ( $P = 0.08$ ), and spruce had the lowest AOA

**Table 2** Mean values (followed by standard error) for mineral soil chemistry parameters among tree species: pH, total C, total N, C:N ratio

Letters indicate significant differences in least square means of tree species effects (ash, beech, lime, maple, oak, spruce,  $df = 5$ ) following Analysis of variance (Type III)

Species	pH	C%	N%	C:N
Tree species				
P	< 0.001	< 0.001	< 0.001	< 0.001
Ash	4.29 (0.19)a	3.08 (0.14)b	0.26 (0.01)b	12.0 (0.1)b
Beech	3.49 (0.04)bc	3.38 (0.34)b	0.24 (0.02)b	14.2 (0.4)b
Lime	4.23 (0.12)a	3.65 (0.32)b	0.26 (0.02)b	13.8 (0.5)b
Maple	4.36 (0.09)a	3.10 (0.22)b	0.24 (0.01)b	13.0 (0.2)b
Oak	3.60 (0.05)b	4.87 (0.78)b	0.33 (0.04)b	14.2 (0.5)b
Spruce	3.32 (0.06)c	7.77 (0.05)a	0.42 (0.08)a	17.0 (1.0)a
Sites				
P	0.003	< 0.001	< 0.001	< 0.001
Matstrup	4.15 (0.13)a	2.70 (0.11)b	0.21 (0.01)b	13.1 (0.2)b
Vallø	3.61 (0.11)c	7.22 (1.24)a	0.42 (0.05)a	16.3 (0.6)a
Viemose	3.76 (0.08)b	3.69 (0.09)b	0.28 (0.01)b	13.4 (0.3)b



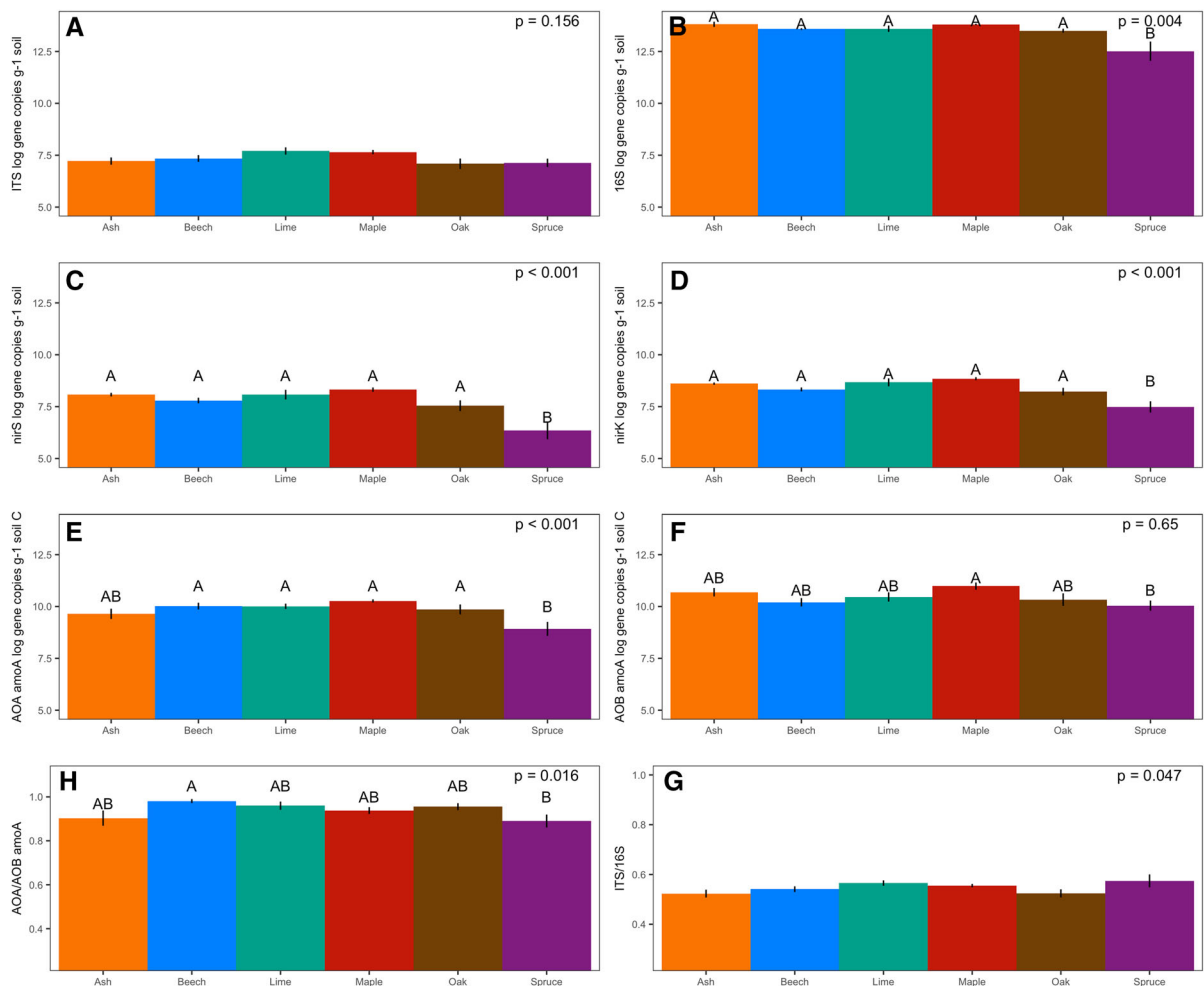


**Fig. 1** Means  $\pm$  standard errors of total ammonium ( $\text{NH}_4^+$ ; **a**) and nitrate ( $\text{NO}_3^-$ ; **b**) concentrations ( $\text{mg N kg}^{-1}$  soil C), and net rates of ammonification (**c**) and nitrification (**d**), gross ammonification (**e**), gross ammonium consumption (**f**), gross nitrification (**g**), and gross nitrate consumption (**h**), averaged across sites ( $\text{mg N kg}^{-1}$  soil C  $\text{day}^{-1}$ ). Data here are per g soil C

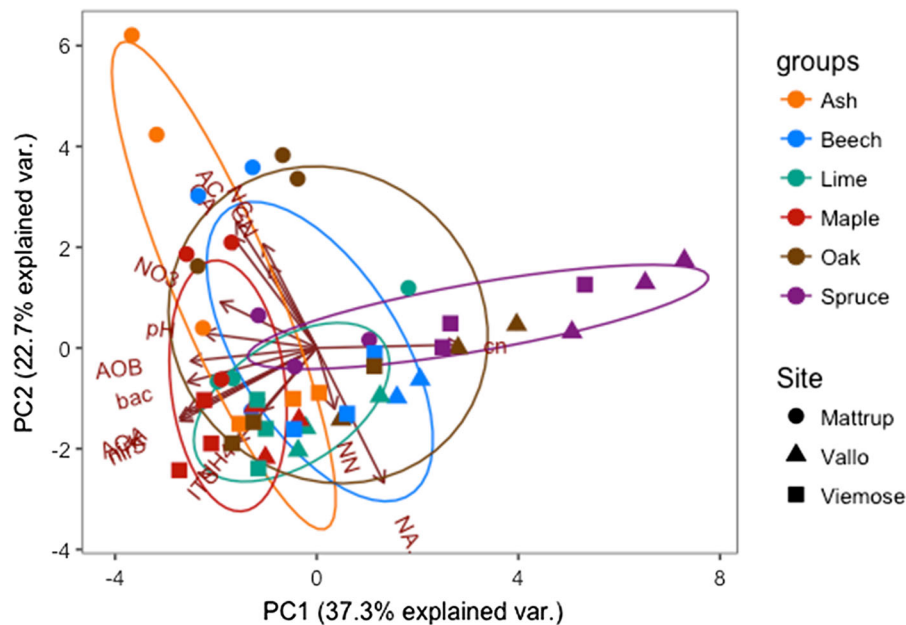
*amoA*:AOB *amoA* ratio, differing significantly from maple (Fig. 2g–h). There were few significant differences among broadleaf species—beech had the lowest abundance of AOB *amoA* but differed significantly only from maple. Tree species effects were quite similar on a per g soil basis, but the spruce effect was stronger for the ITS/16S ratio (Fig. S2H ( $P = 0.047$ ) vs. Fig. 2h ( $P = 0.08$ )) and the lower AOB *amoA*

abundance in spruce on a per g C basis ( $P = 0.01$ ) was less evident on a per g soil basis ( $P = 0.065$ ) as more C under spruce compensated for lower AOB *amoA* per g C (Fig. S2F vs. Fig. 2f).

Principal component analysis (PCA) was used to determine the clustering of all response variables (gene abundances, N transformation rates, and soil characteristics) among the six tree species and the three sites and (Fig. 3). Tree species differed most along principal component 1 which explained 37.3% of the variation and was strongly positively related to C:N ratio and negatively related to pH, and 16S, *amoA* AOA and AOB gene abundances. Spruce (especially at Vallø and also Viemose) was most distinct along PC1 which represented C:N ratio and pH. The three



**Fig. 2** Mean  $\pm$  standard error of the log gene copies/g soil C for *ITS*, *16S*, denitrifying bacteria *nirK* & *nirS*, ammonia-oxidizing archaea and bacteria, and *ITS*:*16S*, and AOA:AOB *amoA* (all data averaged across sites). Data here are per g soil C



**Fig. 3** PCA showing the clustering of all response variables (gene abundances, nitrogen transformation rates, and soil characteristics) with ellipses grouping data by tree species. Data here are per g soil C. Gene abundances includes: bac (for *16S*), *ITS*, *nirS*, *nirK*, AOA and AOB. Nitrogen transformation rates includes: NO<sub>3</sub> (nitrate concentration), NH<sub>4</sub> (ammonium

concentration), NA. (net ammonification), NN (net nitrification), GA (gross ammonification), GN (gross nitrification), GA (gross NH<sub>4</sub><sup>+</sup> consumption), and NC (gross NO<sub>3</sub><sup>-</sup> consumption). Soil characteristics include: pH and carbon to nitrogen ratio

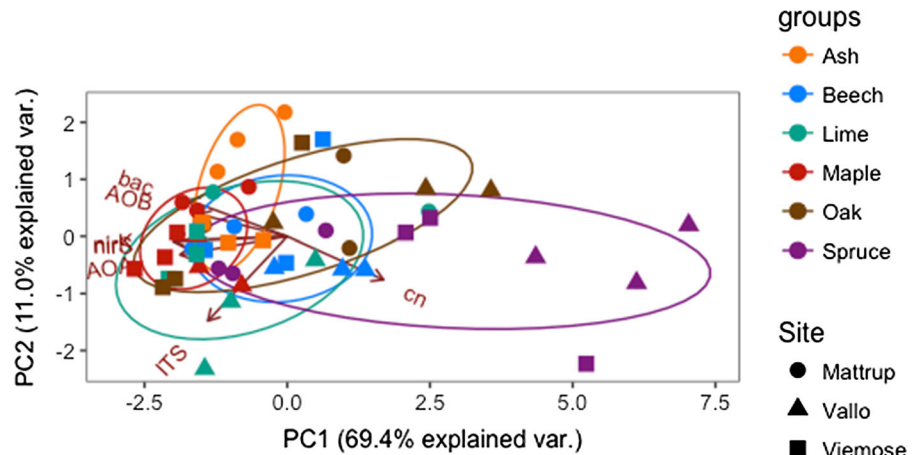
sites differed mostly along principal component 2, which explained 22.7% of the variation, and was positively correlated with gross N transformation rates, and negatively correlated with net nitrification and ammonification rates (see PCA with ellipses grouping by site, Fig. S2). Site Matstrup clustered separately from the other two sites and was related to increased gross rates of production and consumption of ammonium and nitrate. Sites Vallø and Viemose were associated with high net rates of ammonification and nitrification (which were negative), and thus to net immobilization of ammonium and nitrate.

Spruce soils varied little along PC2, and exhibited a much wider variation than broadleaves along PC1 (Fig. 3). Whereas spruce soils at Matstrup clustered with soils from broadleaf species, spruce soil at Vallø was distinct, with high C:N ratios, low pH, and low abundances of bacteria, AOA and AOB (spruce soils from Viemose were intermediate). Ash varied more than other species along PC2 with ash at Matstrup being extreme with high N transformation rates and low fungal ITS. An additional PCA was run to determine if gene abundances alone were well explained by site or

tree species factors. Again, spruce separated from the broadleaf species, most prominently at Vallø along PC1 (Fig. 4), which was strongly positively correlated with C:N ratios and negatively with pH, and ash at Matstrup separated from ash at Viemose. PCA with ellipses grouping by site rather than tree species showed the extreme separation of spruce at Vallø and ash at Matstrup (Fig. S3).

To gain insights into the mechanism(s) underlying the effects of tree species, we used linear mixed effects models to explore linkages between tree species, soil properties, microbial communities and functional genes and N transformation rates. We found a significant interaction between pH and tree species for all the soil microbial abundances except for bacterial *16S* ( $P = 0.2$ ) and AOB, which was close to significance ( $P = 0.06$ ; Supp. Table 1). Microbial abundances increased markedly with increasing pH, but only up to approximately pH 3.8 (Fig. 5), in soils under spruce, oak and beech. The relationships with pH was not as strong in soils under ash, maple, and lime, which all had higher pH (> 3.5). In the plots of spruce, oak and beech that had higher soil pH values

**Fig. 4** PCA showing the clustering of gene abundances and soil characteristics with ellipses grouping data among tree species. Data here are per g soil C. Gene abundances includes: *bac* (for *16S*), *ITS*, *nirS*, *nirK*, AOA and AOB. Soil characteristics include: pH and carbon to nitrogen ratio



(> 3.5), gene abundances were similar to those under ash, maple, and lime. Relationships with C:N ratio revealed the tightest associations to microbial gene abundances for spruce and oak, but little association with C:N ratio in the other tree species (Fig. S4). Soil pH also appeared to underlie differences in nitrification among tree species (Fig. S5). Spruce and oak soils, which had strong relationships between pH and abundance of ammonia oxidizers (AOA and AOB; Fig. 5), also showed positive relationships between pH and gross rates of nitrate production and consumption, although relationships were not quite significant (Fig. S5). These relationships were not evident in soils under the species with soil pH > 3.5 (ash, maple, and lime). The relationships between C:N ratio and N rates or gene abundances were also mainly negative for spruce and oak (Figs. S6 and S7).

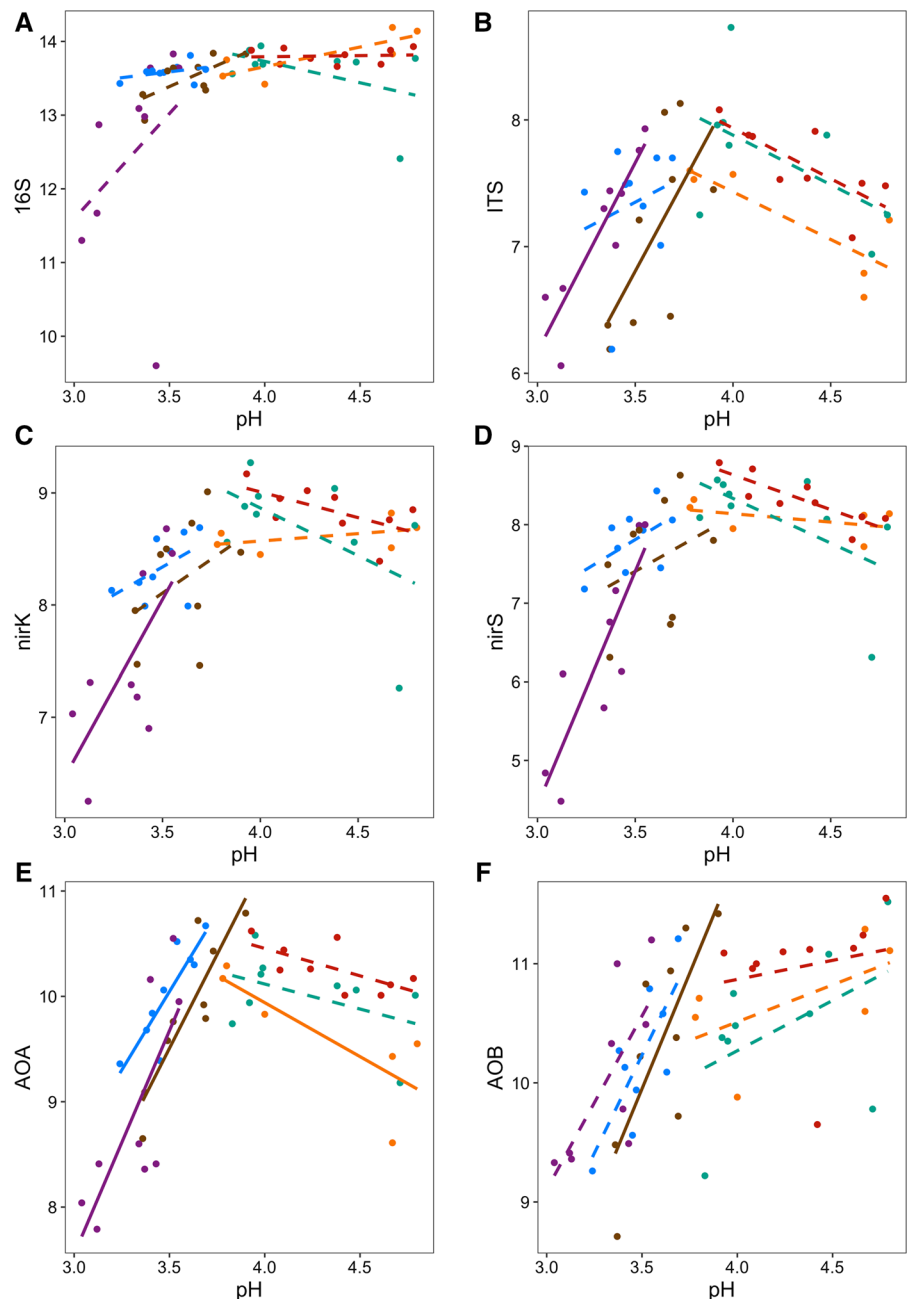
Linear mixed effects models were also used to explore relationships between groups of ammonia oxidizers and gross rates of nitrification (Fig. 6). Positive relationships between abundances of ammonia-oxidizing bacteria and gross rates of nitrification were most tight for ash, maple and lime, but none were significant. Gross nitrification rates were significantly positively related to abundances of ammonia-oxidizing archaea (AOA) in soils under spruce (Fig. 6,  $P < 0.01$ ) and there were no obvious relationships in other tree species.

## Discussion

Our findings from these common garden experiments provide evidence that: (1) tree species influenced N cycling processes and the associated microbial communities in these soils, (2) the effects were primarily driven by differences in soil pH and C:N ratio and attendant effects on microbial communities and/or the expression of genes associated with particular N-cycling processes, and (3) the effects of tree species were modulated by properties of the soils. In particular, the higher pH of site Matstrup, attributable to its previous agricultural land use, appeared to mitigate the expression of ‘the spruce effect’, which was evident at the two (previously forested) sites with lower soil pH.

Soils under Norway spruce were the most distinct, with the lowest rates (per g C) of ammonification processes and gene abundances of bacteria (*16S*), ammonia-oxidizers (both AOA and AOB) and denitrifiers (both *nirS* and *nirK*), and highest (but not significantly different) fungal:bacterial ratio (although spruce soils had relatively high rates of gross nitrification and  $\text{NO}_3^-$  consumption). Along with the high C:N ratio of soil under spruce, these findings are consistent with the characterization of spruce as creating a relatively N-poor environment (Vesterdal et al. 2008, Reich et al. 2005, Oostra et al. 2006). Spruce topsoil at these sites were also associated with low pH and exchangeable base cations, higher Al and Na concentrations and lower moisture conditions relative to the soils under broadleaf species, which contributed to low abundance of burrowing earthworms, and accumulation of organic matter, C and N

**Fig. 5** Linear relationships between pH and gene abundances (log copies/ $\mu$ g soil) for bacterial and archaeal *16S* (a), fungal *ITS* (b), *nirK* (c), *nirS* (d), AOA (e), and AOB (f) between the six tree species (averaged among the three sites). Note the y-axis scale shifts between the six different genes. Colours indicate species, as in previous figures: orange are ash, purple are beech, light green are lime, red are maple, blue are oak, and dark green are spruce. Data here are per g soil C

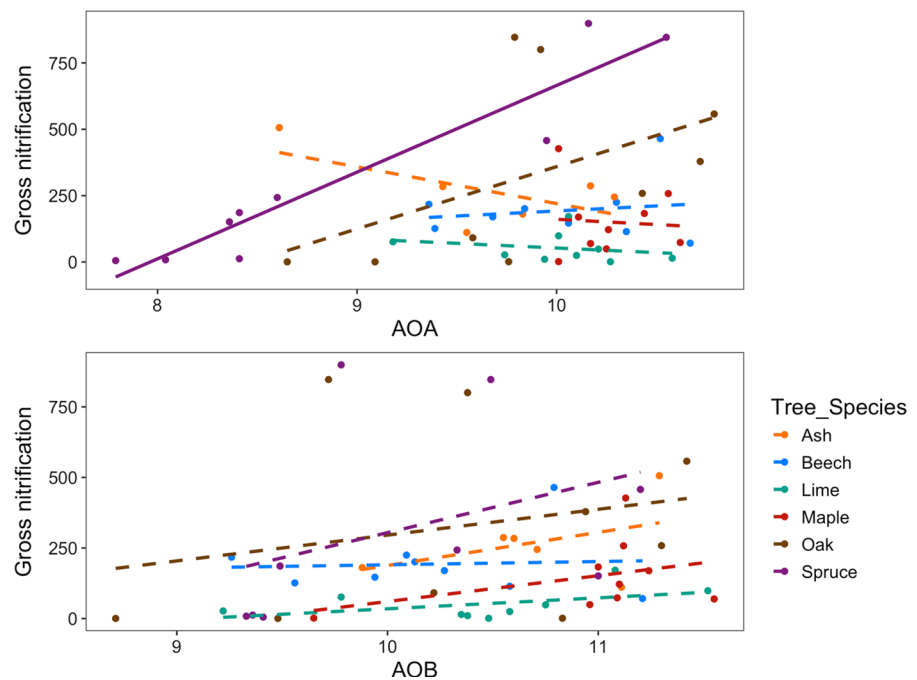


in forest floors rather than in soil in spruce plots (Schelfhout et al. 2017; Vesterdal et al. 2008, 2012). The positive relationships between gross nitrification rates and gene abundances of AOA in soils under spruce indicate that nitrification under this species is linked to ammonia-oxidizing archaea (Fig. 6). This is consistent with other evidence that AOA dominate

nitrification processes in acidic soils (Nicol et al. 2008; Szukics et al. 2012; Hu et al. 2013).

Carbon concentrations were higher in soils under spruce than under broadleaves, so N transformation rates and gene abundances were standardized per g soil C to distinguish between effects resulting from differences in N-cycling and those related only to the amount of organic matter. To some extent the lower

**Fig. 6** Gross nitrification ( $\text{mg N kg}^{-1}$  soil  $\text{C day}^{-1}$ ) as related to *amoA* AOA and AOB gene abundances (log copies/ $\mu\text{g}$  soil C). Colours indicate species, as in previous figures: orange are ash, purple are beech, light green are lime, red are maple, blue are oak, and dark green are spruce



quality of spruce organic matter per g C (lower  $\text{NH}_4^+$  and  $\text{NO}_3^-$  per g C and lower AOB *amoA* per g C) was compensated by the higher concentration of C in spruce topsoils. However, the response of N transformation rates and gene abundances to tree species or tree species-mediated pH and C:N ratios were only marginally different between the two response units. This suggests that the greatest influence occurs via the nature of organic matter produced by the studied tree species rather than via tree species influence on mineral soil C concentrations.

Although there were few significant differences in the measured parameters among the five broadleaf species, lime, ash and maple were distinct in having significantly higher pH ( $> 4.0$ ), which was associated with higher gene abundances of bacteria and archaea (*16S*), denitrifiers (*nirS* and *nirK*) and ammonia oxidizers (*amoA* AOA and AOB); Fig. 5). These findings are consistent with characterization of these species as “mull-forming hardwoods” (Neirynck et al. 2000), which have rapid leaf litter decomposition (Vesterdal et al. 2012) and create conditions of relatively high pH and exchangeable base cations and low Al (Langenbruch et al. 2012; De Schrijver et al. 2007). These conditions promote activity of burrowing earthworms (Reich et al. 2005), formation of mull soils (Schelfhout et al. 2017), and high

bacterial:fungal ratio (Rousk et al. 2009). Soils under oak and beech were generally intermediate to those of spruce and the ‘mull-forming’ species, consistent with their classification as moder-forming tree species (Neirynck et al. 2000) and their intermediate status in terms of decomposition rate, leaf litter base cations, C:N ratio (Vesterdal et al. 2008, 2012) and pH (Schelfhout et al. 2017).

The three species clusters were less distinct in rates of N transformations: gross ammonium production and consumption were lower than expected under lime, and gross nitrate production and consumption did not conform to our expectations that rates would be highest under ash, maple and lime and lowest under spruce, even though the gene abundance of ammonia oxidizers were higher under these species. This discrepancy indicates that the prevalence of nitrification in soil might be better surmised through assessment of functional gene abundances than through laboratory measures of N transformation rates. The distinctly lower ammonium production and consumption rates under lime than the other ‘mull-forming hardwood’ species are also interesting in that lime, like Norway spruce, oak and beech, is an ectomycorrhizal-associated (ECM) species while ash and maple are arbuscular mycorrhizal-associated (AM) species (Lang et al. 2011). In a recent meta-analysis, Lin et al.



(2017) found that inorganic N concentrations, net N mineralization and nitrification rates were all higher in AM than in ECM forests, indicating ‘mineral’ N economy in AM but ‘organic’ N economy in ECM trees. In contrast to influences on pH, the influences of tree species on N-cycling processes might be more closely tied to their mycorrhizal associations and thus the mycorrhizae-associated nutrient economy (MANE) framework proposed by Phillips et al. (2013). In the MANE framework, high nitrification rates under AM species are explained by their nutrient and base-rich, fast-decomposing litter, which releases ample amounts of ammonium and maintains a relatively high soil pH all of which stimulate nitrifying bacteria. The high litter nutrient concentrations (Vesterdal et al. 2012), high soil pH and higher gene abundance of ammonia oxidizers in soil beneath AM-associated tree species (maple and ash) are indicative of an inorganic N economy.

The positive relationships of pH with nitrification process rates and microbial gene abundances indicate a strong controlling effect on N cycling processes, but only up to a threshold (e.g. pH 3.5–3.8). Above this threshold, the relationships disappeared, indicating that influences of other factors became important once the constraint of low pH was alleviated. Only soils under spruce, oak and beech showed this relationship, as they were the only species that acidified the soil sufficiently to lower the pH below 3.8. The same species did not generate this effect at Mattrup—the former agricultural site—presumably because the soil pH and exchangeable base cations were buffered by previous additions of lime. At Mattrup, spruce clustered more closely with the broadleaf species and away from spruce at the previously forested sites in the PCA. At this site, buffering of soil pH appeared to prevent the expression of “the spruce effect” by maintaining soil pH at levels conducive to earthworms and attendant effects of their burrowing activities. A similar lack of expression of mull-forming hardwoods would be expected in soils that are inherently acidic—unless they are close enough to the threshold that the influence of these hardwoods could push them into the range where their characteristic effects can be expressed. Indeed, Gurmesa et al. (2013) noted that broadleaf tree species did not generate mull soils on sites with coarse-textured, acidic and nutrient-poor soils. Similar to our study, van Oijen et al. (2005) found tree species effects to be most pronounced at the

“acidification-sensitive” site and least on the base-rich site. It appears therefore that tree species effects on soil are least pronounced in extreme conditions such as highly acidic or neutral soils, where their influences are insufficient to initiate the shift to another state, but are more pronounced at intermediate sites such as the two old forest sites. Soil C:N ratio was also significantly (negatively) related with gene abundances and N cycling rates in spruce and oak (Figs. 5, S4–S6). We conclude that soil C:N ratio and pH were important factors determining the context-dependency of tree species effects. Identification of other ecological thresholds such as soil texture or moisture regime might assist in understanding the context-dependency of tree species effects and predicting the conditions under which they will be expressed.

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